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# Expression and regulation by insulin of low-density lipoprotein receptor-related protein mRNA in human skeletal muscle

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**Abstract**

Evidence suggests that increased hydrolysis and/or uptake of triglyceride-rich lipoprotein particles in skeletal muscle can be involved in insulin resistance. We determined the steady state mRNA levels of the low-density lipoprotein-related receptor (LRP) and lipoprotein lipase (LPL) in skeletal muscle of eight healthy lean control subjects, eight type 2 diabetic patients and eight nondiabetic obese individuals. The regulation by insulin of LRP and LPL mRNA expression was also investigated in biopsies taken before and at the end of a 3 h euglycemic hyperinsulinemic clamp (insulinemia of about 1 nM). LRP mRNA was expressed in human skeletal muscle ($1.3 \pm 0.1$ amol/μg total RNA in control subjects). Type 2 diabetic patients, but not nondiabetic obese subjects, were characterized by a reduced expression of LRP ($0.8 \pm 0.2$ and $1.3 \pm 0.3$ amol/μg total RNA in diabetic and obese patients, respectively; $P < 0.05$ in diabetic vs. control subjects). Insulin infusion decreased LRP mRNA levels in muscle of the control subjects but not in muscle of type 2 diabetic and nondiabetic obese patients. Similar results were found when investigating the regulation of the expression of LPL. Taken together, these results did not support the hypothesis that a higher capacity for clearance or hydrolysis of circulating triglycerides in skeletal muscle is present during obesity- or type 2 diabetes-associated insulin resistance.



*Keywords:* Gene expression; Insulin resistance; Lipoprotein lipase; Triglyceride; Hyperinsulinemic clamp; RT-PCR

## 1. Introduction

Evidence has been accumulated suggesting that increased lipid availability in skeletal muscle is a cause of insulin resistance in obesity and in type 2 diabetes [1,2]. Based on the initial finding by Randle et al. [3], it has been postulated that the elevation of circulating free fatty acid concentrations, as frequently observed in obese and in diabetic patients, was one of the culprits in the reduced action of insulin [4–6]. A number of observations also suggest that triglyceride availability and hydrolysis in skeletal muscle could be involved in insulin resistance [2]. For instance, infusion of lipid emulsions, composed of triglycerides, reduced insulin action both in humans and animal models [5–8]. However, most of the infused lipids combine with circulating apoproteins to form lipoproteins that are subsequently cleared from the circulation [9]. It has also been demonstrated that high fat feeding induces insulin resistance [10,11]. Dietary fat generally forms chylomicrons that are hydrolyzed by lipoprotein lipase (LPL) in adipose tissue and skeletal muscle capillaries [12]. Thirty-five years ago, it was reported that chylomicrons could inhibit glucose uptake in rat heart [13]. Furthermore, overexpression of LPL in mice skeletal muscle leads to intramuscular lipid accumulation and insulin resistance, without any increase in plasma free fatty acid [14,15]. In humans, recent data also clearly demonstrated that accumulation of intramyocellular triglycerides is strongly associated with insulin resistance [16,17]. All these results therefore suggest that transfer of lipids from

*Abbreviations:* LRP, low-density lipoprotein receptor-related protein; LPL, lipoprotein lipase; RT-cPCR, reverse transcription competitive polymerase chain reaction; BMI, body mass index; NEFA, non-esterified fatty acid

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triglyceride-rich lipoprotein particles to skeletal muscle can be involved in lipid-induced insulin resistance.

The low-density lipoprotein (LDL) receptor-related protein (LRP) is a large multiligand receptor that belongs to the LDL receptor gene family [18] and that has been shown to play a role, in cooperation with the LDL receptor, in the removal of chylomicron remnants from the circulation [19]. In rat adipose tissue, insulin up-regulates LRP gene expression, and this effect is thought to be involved in the stimulation by insulin of the endocytic uptake of remnant lipoproteins by adipocytes [20]. In addition, fed rats expressed more LRP and accumulated significantly more chylomicrons in adipose tissue than did fasted animals [20]. In adipose tissue, insulin and feeding also increase the expression and activity of LPL, the enzyme that catalyses the hydrolysis of triglycerides and thus controls the rate of entrance of fatty acids into fat cells [12]. Therefore, in a postprandial situation, a coordinate stimulating action on LRP and LPL could lead to an increased uptake of triglycerides and fatty acids from remnant lipoproteins in adipocytes.

Little data is available regarding the regulation of lipid uptake in skeletal muscle and its relationship with insulin resistance in humans. The expression and the regulation of LRP in human skeletal muscle is not known, and only a few studies have investigated the expression of muscle LPL in pathological conditions of insulin resistance [21–23]. As discussed above, increased availability and uptake of lipids from triglyceride-rich lipoproteins could be involved in fat accumulation within the muscle cells. However, it is not clear whether the disturbances in lipid metabolism in skeletal muscle result from elevated circulating levels of lipids or from higher hydrolysis and clearance of plasma lipoproteins and triglycerides in skeletal muscle. According to this latter hypothesis, it might be expected that the expression and activity of LPL and other proteins involved in lipid transfer, such as LRP, are increased in skeletal muscle of insulin-resistant patients. The aim of this study was therefore to determine LRP and LPL mRNA expression in skeletal muscle of insulin-resistant patients. In addition, we have investigated the in vivo regulation by insulin of the expression of these two genes in muscle from healthy lean controls, nondiabetic obese subjects and type 2 diabetic patients.

## 2. Materials and methods

### 2.1. Subjects

Twenty-four subjects (eight lean, eight obese nondiabetic and eight type 2 diabetic subjects) who participated in a study aiming to investigate the regulation of gene expression by insulin in human tissues [24,25], were selected for the present work. Their characteristics are presented in Table 1. None of the healthy lean subjects had a familial or personal history of diabetes, obesity or hypertension. None of the obese subjects had impaired glucose tolerance as assessed by classical oral glucose tolerance test (2 h plasma glucose concentration below 7.8 mM following a 75 g oral glucose test, according to the revised American Diabetes Association criteria). All participants gave their written consent after being informed of the nature, purpose and possible risks of the study. The experimental protocols were approved by the Ethical Committees of the Hospices Civils de Lyon (Lyon, France).

Table 1
Characteristics of the subjects

| | Lean | Obese | Type 2 diabetic |
|---|---|---|---|
| *n* | 8 | 8 | 8 |
| M/W | 2/6 | 1/7 | 4/4 |
| Age (years) | 46 ± 4 | 43 ± 5 | 52 ± 2 |
| BMI (kg/m$^2$) | 23 ± 1 | 35 ± 2[a] | 30 ± 1[a] |
| Glucose (mM) | 4.9 ± 0.9 | 5.0 ± 0.1 | 11.8 ± 1.1[a,b] |
| Insulin (pM) | 41 ± 5 | 83 ± 21[c] | 55 ± 10 |
| Cholesterol (mM) | 5.5 ± 0.3 | 5.6 ± 0.3 | 5.0 ± 0.4 |
| NEFA (μM) | 436 ± 42 | 629 ± 61[c] | 652 ± 53[a] |
| Triglycerides (mM) | 0.8 ± 0.1 | 1.2 ± 0.1[c] | 1.3 ± 0.1[c] |
| *Clamp study*[d] | | | |
| Glucose (mM) | 4.4 ± 0.2 | 4.4 ± 0.1 | 5.0 ± 0.1[e] |
| Insulin (pM) | 1015 ± 67 | 1157 ± 157 | 1153 ± 160 |
| Glucose disposal rate (mg/kg/min) | 9.5 ± 1.5 | 4.3 ± 0.8[a] | 4.1 ± 0.5[a] |

Plasma metabolite and hormone concentrations were determined after an overnight fast.
[a] $P<0.01$ vs. lean subjects.
[b] $P<0.01$ vs. obese subjects.
[c] $P<0.05$ vs. lean subjects.
[d] Measured during the last hour of the clamp.
[e] $P<0.05$ vs. obese subjects.

### 2.2. Euglycemic hyperinsulinemic clamp

To investigate insulin action on gene expression, all subjects were studied using the euglycemic hyperinsulinemic clamp technique after an overnight fast. The clamp was performed as previously described [24,26], with a rate of insulin infusion of 450 pmol/m$^2$ body surface area/min for 3 h. Serum insulin concentration was measured by commercial radioimmunoassay, and serum metabolite concentrations were determined by enzymatic assays [26].

### 2.3. Skeletal muscle biopsies

Muscle samples (vastus lateralis) were taken under local anesthesia (2% Lidocain without adrenaline), 3 h before and at the end of the hyperinsulinemic clamp period by percutaneous biopsies using a Weil Blakesley plier, as previously described [24,25]. The average weight of the muscle samples were 51 ± 4 mg wet weight ($n=48$) with no difference between samples from lean, obese and type 2 diabetic subjects or before and after clamp. Possible contamination of the muscle biopsies by fat cells was unlikely since the

expression level of leptin mRNA was found to be below the detection limit of a very sensitive RT-competitive PCR (RT-cPCR) assay [27] in these muscle samples (data not shown).

### 2.4. Total RNA preparation

Tissue samples were crushed in liquid nitrogen, and total RNA was prepared according to a modified procedure of Chomczynski and Sacchi [28]. Average yields of total RNA were 25 ± 3 μg/100 mg, and were not significantly different in tissues from lean, obese and type 2 diabetic subjects, before or after the clamp. Integrity of the RNA preparations was checked by electrophoresis in agarose gel stained with ethidium bromide. Total RNA solutions were stored at − 80 °C until quantification of LRP mRNA.

### 2.5. Quantification of LRP mRNA

Human LRP mRNA was quantified by RT-cPCR, which consists of the co-amplification of target cDNA with known amounts of a specific DNA competitor molecule added in the same PCR tube [29]. To generate a specific LRP competitor DNA, a 440-bp-long cDNA fragment was obtained by RT-PCR using 5′-TGGAACAGATGGCCATC-GAC-3′ as sense primer (nucleotides +1311 to +1330 of the human LRP cDNA sequence, and 5′-CGAGTTGG-TGGCATAGAGAT-3′ (nucleotides +1731 to +1750) as antisense primer. The competitor was obtained by deleting 30 bp (from nucleotide +1330 to +1360) using a PCR-based mutagenesis strategy as described by Schneeberger and Zeillinger [30]. The LRP competitor fragment (406 bp) was subcloned (pGEM-T, Promega) and the plasmid was purified, carefully quantified and stored at − 20 °C. Working solutions (20 amol/μl to $10^{-3}$ amol/μl) were prepared by serial dilutions in 10 mM Tris–HCl (pH 8.3), 1 mM EDTA buffer.

For the assay of LRP mRNA, the reverse transcription reaction was performed from 0.2 μg of tissue total RNA with 2.5 U of thermostable reverse transcriptase (Tth, Promega, Charbonières, France), in the presence of 15 pmol of LRP-specific antisense primer. The conditions of the reaction have been described in detail previously and warranted optimal synthesis of first-strand cDNA [29]. For the competitive PCR amplification, the reverse transcription medium was added to a PCR master mix (10 mM Tris–HCl pH 8.3, 100 mM KCl, 0.75 mM EGTA, 5% glycerol) containing 0.2 mM deoxynucleoside triphosphates, 5 U of Taq polymerase (Life Technologies, Cergy Pontoise, France), 30 pmol of LRP antisense primer and 45 pmol of LRP sense primer. The sense primer was 5′-labeled with the CY-5 fluorescent dye (Eurogentec, Seraing, Belgium). The final volume was 100 μl and four aliquots of 20 μl were transferred to 0.5-ml microtubes containing 5 μl of a defined working solution of the LRP competitor. After 120 s at 94 °C, the PCR mixtures were subjected to 40 cycles of PCR amplification with a cycle profile including denaturation for 60 s at 94 °C, hybridization for 60 s at 58 °C and elongation for 60 s at 72 °C. The PCR products were separated and analyzed by polyacrylamide gel electrophoresis (6%) using a ALFexpress DNA sequencer (Pharmacia, Upsala, Sweden) and the Fragment Manager software (Pharmacia). The RT-cPCR assay of LRP mRNA was validated using known amounts of an in vitro synthesized LRP RNA fragment (Riboprobe System, Promega), as recommended [29]. LPL mRNA levels were determined using a RT-cPCR assay previously described [26].

### 2.6. Statistical analysis

All data are presented as mean ± S.E. between group comparisons were done using Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U*-test when the ANOVA indicated a significant difference. Wilcoxon's test for paired values was used when comparing mRNA levels before and after clamp. Correlation coefficients were calculated using Spearman's test. $P < 0.05$ was considered statistically significant.

## 3. Results

Under basal conditions, after an overnight fast, LRP mRNA is expressed in human skeletal muscle as shown in Fig. 1. When comparing the mRNA levels of LRP in the muscle of healthy lean control, type 2 diabetic and non-diabetic obese subjects, the Kruskal–Wallis test indicated a tendency for a difference ($P = 0.097$). The non-parametric Mann–Whitney *U*-test showed that type 2 diabetes, but not obesity, was associated with a significant ($P = 0.035$) reduc-

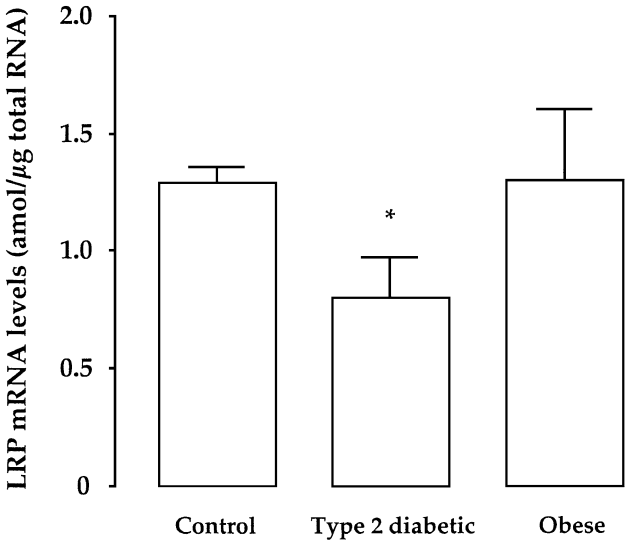


Fig. 1. Basal mRNA levels of LRP in skeletal muscle from lean, obese nondiabetic and type 2 diabetic subjects. The steady state concentrations of LRP mRNA were determined by RT-cPCR in skeletal muscle biopsies taken after an overnight fast in healthy lean subjects, obese nondiabetic and type 2 diabetic patients as indicated under Materials and methods. Data are mean ± S.E. * $P < 0.05$.

tion in the abundance of LRP mRNA (Fig. 1). When the data of the control subjects and the type 2 diabetic patients were analyzed together, there was a trend for a negative correlation between fasting insulinemia and basal LRP mRNA levels ($r = -0.50$, $P = 0.058$). This correlation was not found when the obese subjects were included in the analysis. Skeletal muscle LRP mRNA concentration was not associated with either fasting glycemia, plasma non-esterified fatty acid concentrations, plasma triglyceride concentrations, plasma cholesterol concentrations or insulin-stimulated whole body glucose uptake (data not shown). In the same tissue samples, LPL mRNA expression was significantly lower in type 2 diabetic patients than in control subjects ($14 \pm 3$ vs. $54 \pm 6$ amol/mg of total RNA, $P = 0.006$). In addition, reduced LPL expression was also observed in the muscle of the nondiabetic obese subjects ($23 \pm 5$ vs. $54 \pm 6$ amol/mg of total RNA, $P = 0.04$). There was no correlation between LRP and LPL mRNA levels in skeletal muscle. It should be noted that in contrast to LRP and LPL, the mRNA levels of a number of other genes, such as β-actin, insulin receptor and Glut 4, were not different between groups in muscle preparations from the same type 2 diabetic and obese subjects (data not shown), as previously reported [25].

Hyperinsulinemia for 3 h produced a significant ($P = 0.012$) decrease in LRP mRNA levels in the skeletal muscle of lean control subjects (Fig. 2). In contrast, LRP mRNA expression was not modified during the hyperinsulinemic clamp in the muscle of obese nondiabetic subjects or of type 2 diabetic patients. Similar changes were observed in response to insulin for LPL expression (Fig. 3). LPL mRNA levels were significantly reduced during the hyperinsulinemic clamp in control subjects ($-21 \pm 8\%$, $P = 0.018$), but not in type 2 diabetic and in obese patients.

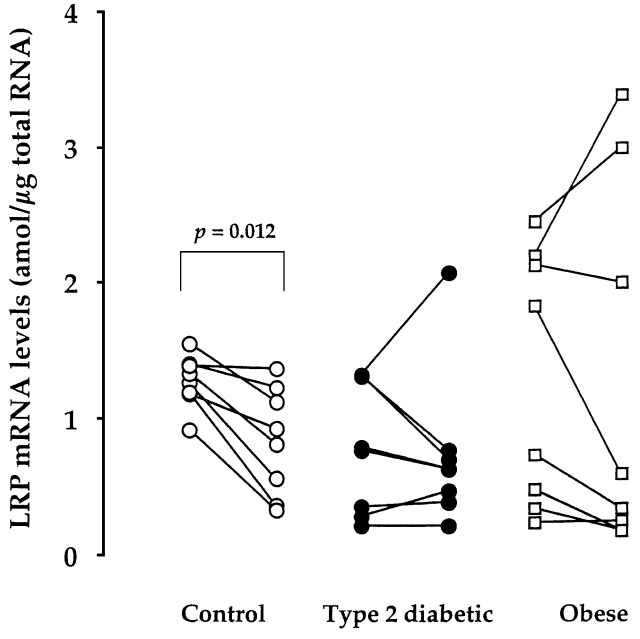


Fig. 2. Regulation of LRP mRNA expression by insulin. Acute 180 min insulin infusion significantly decreased the expression of LRP mRNA in skeletal muscle of lean subjects ($P = 0.012$) but not of obese nondiabetic ($P = 0.575$) or type 2 diabetic subjects ($P = 0.735$).

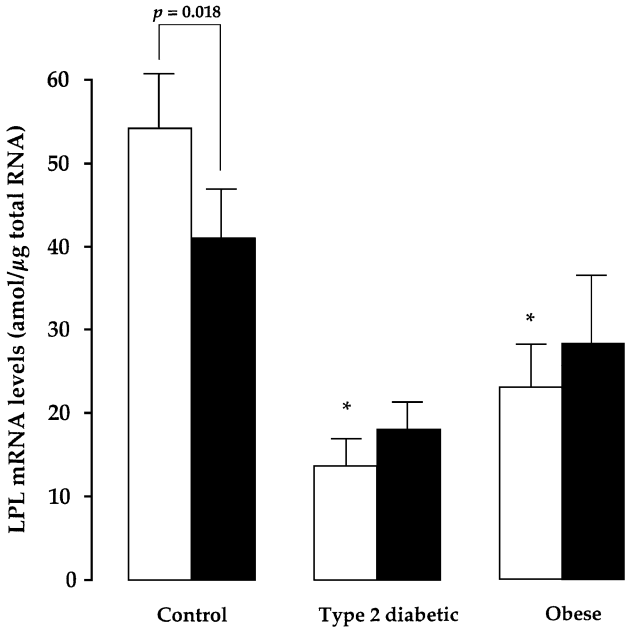


Fig. 3. Regulation by insulin of LPL mRNA levels. The concentrations of LPL mRNA were determined by RT-cPCR in skeletal muscle biopsies taken before (open boxes) and at the end (closed boxes) 3 h of hyperinsulinemic clamp in control subjects, obese nondiabetic and type 2 diabetic patients. Insulin infusion significantly decreased the expression of LPL mRNA in skeletal muscle of lean subjects ($P = 0.018$) but not of obese nondiabetic ($P = 0.345$) or type 2 diabetic subjects ($P = 0.499$). Data are means ± S.E. $^*P < 0.05$ vs. control subjects.

## 4. Discussion

In this study, it was demonstrated that LRP was expressed in human skeletal muscle and that insulin infusion for 3 h reduced LRP mRNA levels in healthy lean subjects. Furthermore, there was an about 2-fold reduction in the abundance of LRP transcripts, and a lack of response to insulin infusion, in the muscle of type 2 diabetic patients. Similar results were observed for LPL mRNA expression.

LRP has been shown to be involved, among other functions [31], in the removal of chylomicron remnants from the circulation [19]. It is mainly expressed in liver and adipose tissue where most of the clearance of chylomicrons occurs [31]. The presence of LRP mRNA, although at rather low levels (about 10-fold less LRP than LPL transcripts) in human muscle and the fact that its expression was regulated by insulin, suggest that LRP protein might be expressed at the membrane of the myocytes and have a biological function. With respect to liver and adipose tissue, skeletal muscles are not considered as major sites of chylomicron removal but evidence suggests that higher targeting of lipoproteins and increased lipid uptake may play a role in the development of insulin resistance [2]. It is indeed well

demonstrated that infusion of lipid emulsions, composed of triglycerides that form lipoproteins in the circulation [9], reduced insulin action both in humans and animal models [5–8]. Furthermore, high fat feeding induces muscle insulin resistance [10,11], and dietary fat generally forms chylomicrons that are hydrolyzed by LPL in adipose tissue and skeletal muscle capillaries [12]. Therefore, an increased capacity for the transfer of lipids from triglyceride-rich lipoprotein particles to the muscle cells can be involved in lipid-induced insulin resistance. We found here that there is no evidence for higher expression of either LRP or LPL, the rate-limiting enzyme for the uptake of fatty acids from circulating triglycerides, in muscle of insulin-resistant subjects. Even more, there was a marked reduction in the mRNA levels of both LRP and LPL in skeletal muscle of type 2 diabetic patients. If the changes in mRNA abundances are translated into a reduction in LRP and LPL proteins and activities, one must thus assume that skeletal muscle of insulin-resistant subjects do not have increased capacity for removal of triglycerides from the circulation. Regarding LPL, the observed reduction in mRNA levels is in keeping with a decreased LPL activity found in muscle of insulin-resistant subjects [21]. For LRP and the other proteins involved in the removal of circulating lipoproteins and the uptake of lipids, further studies are required to verify whether their activity is altered in insulin resistance. Nevertheless, we have recently reported that the mRNA expression of the fatty acid transport protein-1 (FATP-1) is reduced in skeletal muscle of insulin-resistant women [32]. In addition, it has been shown that the uptake of long chain fatty acids in skeletal muscle is reduced in fasting conditions in type 2 diabetic patients [33], and that the uptake of fatty acid across the leg is decreased in women with visceral obesity and insulin resistance [34]. Taken together, these data strongly suggest that insulin resistance is not characterized by higher capacities for the capture and uptake of lipids in skeletal muscle. Therefore, increased availability of triglycerides and fatty acids due to higher circulating concentrations, or altered intracellular lipid metabolism, seem more likely to explain the relationship between lipids and insulin resistance [1,2]. This might in turn down-regulate the expression of the genes coding for the proteins involved in lipid uptake (like LPL and LRP) through mechanisms that are not yet determined.

Previous studies have clearly demonstrated that LRP gene expression was up-regulated by insulin in vivo in rodent adipose tissue [20]. In adipocytes, LRP mRNA increased within minutes after the cells were exposed to physiological concentrations of insulin [20]. In other cells, such as macrophages [35], insulin also increased LRP mRNA levels and LRP activity, in a dose- and time-dependent manner. Moreover, insulin action on LRP expression involved both the PI 3-kinase and Ras/MAPkinase signaling pathways in macrophages [35]. These data demonstrated that LRP gene is a bona fide target gene of insulin. In our study, LRP gene expression also appeared to be regulated during insulin infusion. However, 3 h of hyperinsulinemia reduced LRP mRNA levels in skeletal muscle of the lean control subjects. Similar down-regulation by insulin was observed for LPL mRNA, in agreement with a previous study [26]. Therefore, there is an opposing action of insulin in muscle and in adipocytes on LPL and LRP gene expression. This result is in keeping with the expected biological effects of insulin which promotes an orientation of the flux of lipids to adipose tissue where they are stored and a reduction in lipid utilization in muscles, favoring glucose uptake. Our results showed that the regulation by insulin of the balance between lipid and glucose metabolisms in skeletal muscle also occurs at the transcriptional level. For instance, it has been reported that insulin up-regulates the expression of several genes coding proteins of glucose metabolism, such as Glut 4 [25,26,36,37] or hexokinase II [25,38] and we showed here that insulin down-regulates the expression of genes involved in the uptake of lipids, such as LPL and LRP.

The effect of insulin on LPL and LRP expression is altered in muscle from nondiabetic obese subjects and type 2 diabetic patients. Defective regulation by insulin of gene expression has been previously documented in diabetic patients [25,36–38]. We have demonstrated that the altered regulation of hexokinase II, p85αPI 3-kinase and the transcription factor SREBP1c was encountered in type 2 diabetic patients and not in nondiabetic obese subjects, suggesting that type 2 diabetes is characterized by specific transcriptional alterations which are not directly linked to insulin resistance [25]. In the same group of subjects, however, the regulation by insulin of other gene, such as Glut 4 [25], was found to be impaired both in type 2 diabetic patients and in nondiabetic obese subjects, suggesting that this defect was likely a consequence of insulin resistance. In this study, the effect of insulin on LRP and LPL mRNA expression was also altered in type 2 diabetic patients and in nondiabetic obese subjects, indicating that the defect, as for Glut 4, probably results from the reduced insulin action in the muscle of these subjects.

In conclusion, this study showed that LRP was expressed in human skeletal muscle and that insulin, in vivo, decreased its mRNA levels in muscle of insulin-sensitive control subjects. In type 2 diabetic patients, the expression levels of muscle LRP mRNA was significantly reduced compared to healthy subjects and the regulation by insulin was altered. Similar results were found for LPL transcripts. Taken together, these results did not support the hypothesis that a higher hydrolysis and/or uptake of triglyceride-rich lipoprotein particles in skeletal muscle can be involved in obesity- and type 2 diabetes-associated insulin resistance.

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